NUCLEIC ACIDS IN CHLOROPLASTS OF A *CHLORELLA* SP.

A. R. Pillay,* Y. T. Tchan† and K. Y. Cho‡

(Plates xiii and xiv)

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Synopsis

Nucleic acids in chloroplasts of *Chlorella* (NM1) are demonstrated by light and electron microscopy. The chloroplasts are positively stained by Feulgen stain. Electron microscopical examination shows fine filamentous fibrils in association with lamellar membranes and ribosomes. The fibrils are linear, single and double looped, and morphologically are not unlike that of DNA. They are distributed throughout the chloroplast matrix and tend to accumulate around the pyrenoid and in areas of low density between the chloroplast lamella. Polysomes are detected at these sites. The fibrils are not observed after treatment with deoxyribonuclease. It is concluded that these fibrils contain DNA and their significance is discussed.

Introduction

Biochemical studies indicate protein synthesis in the chloroplast as distinct from cytoplasmic protein synthesis (Kirk, 1964 ; Ray and Hanawalt, 1965 ; Smillie *et al.*, 1967 ; Selsky, 1967). Such findings agree with the cytological demonstration of RNA and DNA in chloroplasts of plants and algae (Ris and Plaut, 1962 ; Brawerman, 1962, 1963 ; Eisenstadt and Brawerman, 1964 ; Kislev *et al.*, 1965 ; Gunning, 1965 ; Sagen *et al.*, 1965). In *Chlorella* however, there is still no direct demonstration of DNA in the chloroplast although biochemical investigations strongly support its presence (Kirk, 1971). The present paper reports the location and morphology of DNA and RNA in the chloroplast of *Chlorella* (NM1) using light and electron microscopy.

Materials and Methods

Organism and Growth Conditions

A species of *Chlorella*, isolated from a garden soil and designated NM1, was used.

Cells were grown in Erlenmeyer flasks in a defined mineral solution (Tchan, 1959) at pH 6·8 and aerated with CO_2-enriched air. The flasks were placed in a water shaker-bath at 28°C and illuminated by two (60W) incandescent lamps. The intensity of incident light on growth flasks was 50 lux. Samples were taken from two-day old cultures for cytological studies. Preliminary work indicated that the removal of starch grains from the cells was necessary to facilitate the detection of fine structure. This was achieved by keeping actively growing two-day old culture in the dark without agitation for 12 hours at room temperature (22°C).

Light Microscopy

Cells were fixed in alcohol-acetic acid (3 : 1) for 10 minutes. They were mounted on glass slides, washed with absolute ethanol and then brought to water

* Mauritius Sugar Industry Research Institute, Mauritius.

† Department of Microbiology, University of Sydney, New South Wales, 2006.

‡ Linnean Macleay Lecturer in Microbiology, Department of Microbiology, University of Sydney, New South Wales, 2006.

through a graded series of alcohol. Some slides were treated for 15 minutes in N HCl at 60°C, and stained with Feulgen's reagent for 90 minutes in darkness. Other slides, after hydrolysis treatment, were stained with a Feulgen-fluorescent dye for 60 minutes in the dark. Control slides were stained without hydrolysis. All slides were washed in distilled water, and bleached in a mixture of sodium bisulphite : N HCl, and distilled water (1 : 3 : 3 w/v/v) for 10 minutes with two changes. The slides were washed with tap water and mounted in glycerine.

Preparations stained with the fluorescent dye were observed by phase contrast and fluorescence microscopy using a 100× oil immersion objective. Fluorescence illumination was provided by an illuminator equipped with a HB200 high-pressure mercury arc lamp and a 3 mm BG12 excitation filter, and K530 barrier filter. The phase and fluorescence images were recorded on Kodak Super XX film with an initial magnification of ×600 and a final enlargement to ×3,350.

Electron Microscopy

A. Cells were prefixed at room temperature in 2·5% glutaraldehyde in sodium cacodylate buffer (0·045M, pH 6·2) for two hours, washed in the buffer for 24 hours (three changes) and followed by :

(i) post-fixation in 1% OsO_4 in Michaelis buffer (pH 6·0) overnight at room temperature (Pease, 1964). The cells were washed three times in a 3-fold dilution of the buffer and then suspended in a small amount of 1·5% agar (50°C) ; or

(ii) post-fixation in freshly prepared 0·5% unbuffered $KMnO_4$ solution for 10 minutes then washed five times in a 3-fold dilution of Palade's (1952) acetate buffer (pH 6·2) and suspended in a small amount of 1·5% agar (50°C).

Digestion by Deoxyribonuclease

B. Cells were fixed in 10% formalin in veronal acetate buffer (Pease, 1964) (pH 6·2) for 10 minutes, washed in distilled water and digested with 0·001% deoxyribonuclease I (Sigma) in veronal acetate buffer at 40°C for four hours.

The control sample was incubated in buffer only. After treatment cells were prefixed in glutaraldehyde and post-fixed in potassium permanganate solution as in A (ii), above.

The small agar blocks from fixations A and B were stained with 0·5% uranyl acetate buffered solution for two hours. Dehydration was carried out at five-minute intervals in 30%, 50%, 70% and 80% ethanol followed by 10 minutes in 90% and two changes in 100% ethanol. The blocks were cut into approximately 2 mm pieces, infiltrated in three changes of araldite for one hour each and overnight at room temperature. Polymerisation was realised in fresh araldite at 60°C for 36–48 hours. Sections were cut on a Porter Blum ultra-microtome MT–2 using glass knives and collected in nitrocellulose coated grids. After staining with lead citrate (Reynolds, 1963), sections were examined and photographed with a Philips EM200 or a Siemens Elmiskop I operating at 60 or 80 kV.

Results

Light Microscopy

In Feulgen stained cells, the chloroplast gives a colour reaction of about the same intensity as the nucleus (Pl. XIII, Fig. 1). A comparison of the internal structures of cell shown by Feulgen-fluorescent staining with those observed in phase contrast microscopy (Pl. XIII, Figs 2 and 3) clearly demonstrated the presence of DNA positive sites in the chloroplasts.

Electron Microscopy

Pl. XIII, Fig. 4 shows the presence of starch around the pyrenoid and in areas between the lamellae of the chloroplast. Pl. XIII, Fig. 5 was printed for maximum contrast to demonstrate areas of low electron density. These areas are not bounded by membranes and contain fibrils (Pl. XIII, Fig. 5). They are present in the vicinity of the pyrenoid region and randomly distributed within the chloroplast (Pl. XIII, Fig. 5).

In OsO_4 fixed cells the low density areas contain conspicuous fine fibrils only prior to deoxyribonuclease treatment (Pl. XIII, Fig. 7 ; Pl. XIV, Fig. 1). These fibrils are removed after treatment by deoxyribonuclease (Pl. XIII, Fig. 6). At a higher magnification, unattached filaments, and numerous DNA structures attached to the lamellar membranes are well resolved in linear, single and double looped configuration.

In sections of chloroplasts a number of DNA-containing areas occur in the vicinity of the pyrenoid and between the lamellae (Pl. XIII, Figs 5 and 7 ; Pl. XIV, Fig. 1). Their distribution is much more conspicuous than that reported by Ris and Plaut (1962) for *Chlamydomonas*. Unlike the brown algal (Gibbs, 1967, 1968 ; Bisalputra and Burton, 1969 ; Bisalputra and Bisalputra, 1969) they lack regular patterns.

Ribosomes and polyribosomes are frequently seen in close proximity or adhering to fibrils near the lamellar membranes. Free ribosomes are in the vicinity of pyrenoid and in the stroma regions between the lamellae (Pl. XIV, Figs 1 and 2).

Discussion

Direct fixation with OsO_4 or $KMnO_4$ usually fails to preserve adequately the fine structures within the chloroplast (Gunning, 1965). $KMnO_4$ is reported to extract RNA but stabilises DNA as a branching network of fibrils (Luft, 1956 ; Bradbury and Meek, 1960 ; Nass and Nass, 1963 ; Nass *et al.*, 1965). OsO_4 can also result in leaching of proteins. It is not clear whether DNA fibrils are restricted to areas of low electron density in the chloroplast or more widely dispersed and obscured by ribosomes. It is known that direct $KMnO_4$ fixation removes ribosomes (Hayat, 1970). The present study shows that $KMnO_4$ could be used as a suitable post-fixative after the stabilisation of RNA and proteins by gluteraldehyde.

The absence of fibrils in the low density areas of the ground substance in the chloroplast after deoxyribonuclease treatment strongly suggests that these fibrils contain DNA, and this is in accord with the appearance of the chloroplasts before treatment and the positive Feulgen staining reaction (Pl. XIII, Figs 1, 2 and 3). The disappearance of ribosomes and the preservation of fibrils by $KMnO_4$ fixation also lends further support.

Conclusion

The present cytological investigation clearly demonstrated the presence of DNA in chloroplast of *Chlorella* (NM1). It exists as a number of short single units (Pl. XIV, Fig. 2). They are not restricted to the low electron density areas but are also found in electron dense areas where their presence may sometimes be obscured by ribosomes. This suggests a wider distribution of the DNA chloroplast. However, a few areas containing DNA appear to be randomly distributed through the chloroplast matrix and around the pyrenoid region. These areas may represent sites of greater activity in the chloroplast. Thus the early biochemical evidence is now supported by direct cytological observation.

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EXPLANATION OF PLATES

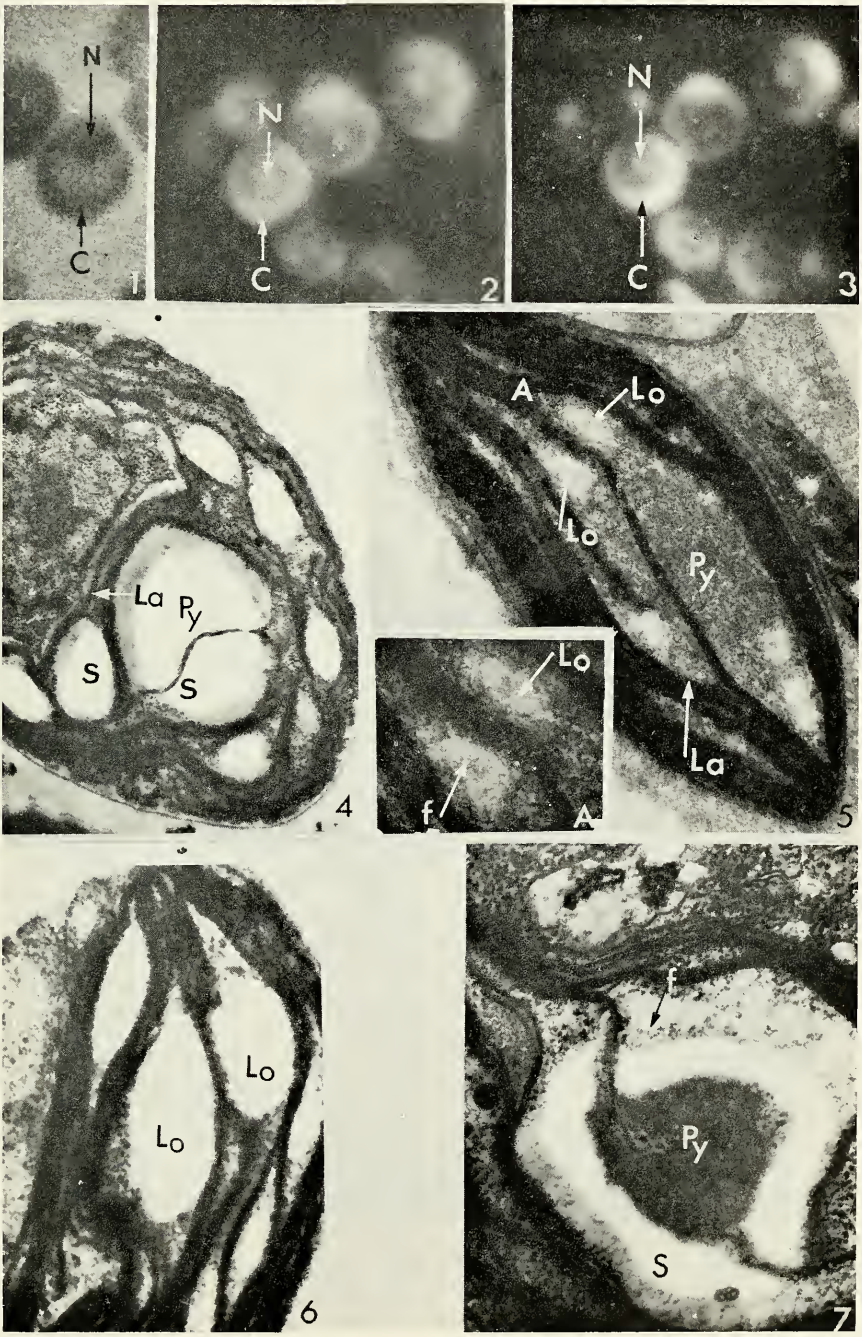
Plate xiii

Fig. 1. Feulgen stained *Chlorella* NM1 cell, showing nucleus (N) and chloroplast (C). × 3,350.

Fig. 2. Feulgen-fluorescent dye stained *Chlorella* NM1 cells examined by phase contrast microscopy. Chloroplast (C), nucleus (N). × 3,350.

Fig. 3. The same cells as in Fig. 2 examined by fluorescent microscopy. Note fluorescence in regions identical to those identified as chloroplast (C) and nucleus (N) in Fig. 2. × 3,350.

Fig. 4. *Chlorella* NM1. Note starch (S) in pyrenoid area (Py) and starch granules (S) scattered in areas between lamellae (La) of chloroplast. Glut. OsO_4. × 22,100.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